Protein trafficking in *Plasmodium falciparum*-infected red cells and impact of the expansion of exported protein families

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SUMMARY

Erythrocytes are extensively remodelled by the malaria parasite following invasion of the cell. *Plasmodium falciparum* encodes numerous virulence-associated and host-cell remodelling proteins that are trafficked to the cytoplasm, the cell membrane and the surface of the infected erythrocyte. The export of soluble proteins relies on a sequence directing entry into the secretory pathways in addition to an export signal. The export signal consisting of five amino acids is termed the *Plasmodium* export element (PEXEL) or the vacuole transport signal (VTS). Genome mining studies have revealed that PEXEL/VTS carrying protein families have expanded dramatically in *P. falciparum* compared with other malaria parasite species, possibly due to lineage-specific expansion linked to the unique requirements of *P. falciparum* for host-cell remodelling. The functional characterization of such genes and gene families may reveal potential drug targets that could inhibit protein trafficking in infected erythrocytes. This review highlights some of the recent advances and key knowledge gaps in protein trafficking pathways in *P. falciparum*-infected red cells and speculates on the impact of exported gene families in the trafficking pathway.

Key words: *Plasmodium falciparum*, erythrocyte remodelling, protein trafficking, exported proteins, gene family expansion.

INTRODUCTION

Malaria is an infectious vector-borne disease responsible for significant global morbidity and mortality (Snow *et al*. 2005). The vast majority of the fatal cases of malaria are caused by *Plasmodium falciparum*, although *Plasmodium vivax*, which is itself responsible for huge morbidity (Mendis *et al*. 2001), may also, although rarely, be severe and fatal (Kochar *et al*. 2005; Rogerson and Carter, 2008; Suwanarusk *et al*. 2008; ter Kuile and Rogerson, 2008; Tjitra *et al*. 2008; Andrade *et al*. 2010). The sequestration of parasitized erythrocytes in the deep vasculature is the main cause of the pathology of severe falciparum malaria. Mature trophozoites and schizonts sequester in the peripheral circulation due to the adhesion of infected erythrocytes to endothelial cells (cytoadherence) and with uninfected erythrocytes (rosetting) leading to significantly impaired blood circulation (Miller *et al*. 1994). Infected erythrocytes also become more rigid and adhere to different cell types (Raventos-Suarez *et al*. 1985).

The malaria parasite modifies its host-cell environment, presumably to enhance its own survival, and this leads to pathological consequences for the host. While all stages of the parasite modify their host cell to a certain extent, infected erythrocytes are subject to extensive modifications that are vital for parasite survival (Miller *et al*. 1994). Human erythrocytes lack protein trafficking machinery, so, following invasion, *P. falciparum* first has to establish a trafficking pathway to export various proteins to the surface of the infected cell.

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HOST-CELL MODIFICATION AND PROTEIN TRAFFICKING

The modification of the erythrocyte from a free-flowing and essentially non-adhesive cell to one that is capable of adhering to endothelial cells and non-infected erythrocytes (Miller *et al*. 1994) highlights the dramatic modification that occurs following the invasion of the malaria parasite. Major changes that occur in the infected erythrocyte include the formation of small protrusions on the surface of the cell (knobs), alterations in ion channel behaviour (Decherf *et al*. 2004; Staines *et al*. 2007), the formation of novel channels for nutrient import (Saliba *et al*. 1998; Desai *et al*. 2000; Staines *et al*. 2004), membrane rigidity and cell deformability (Glenister *et al*. 2002) and altered behaviour of infected erythrocytes in the microcirculation (Diez-Silva *et al*. 2012). These modifications occur as a result of the export of various effector proteins in the infected erythrocyte. Mature erythrocytes are devoid of any endogenous vesicle trafficking machinery; therefore, for the parasite to export proteins, it needs to establish its own trafficking pathway. The major obstruction for protein export in the infected

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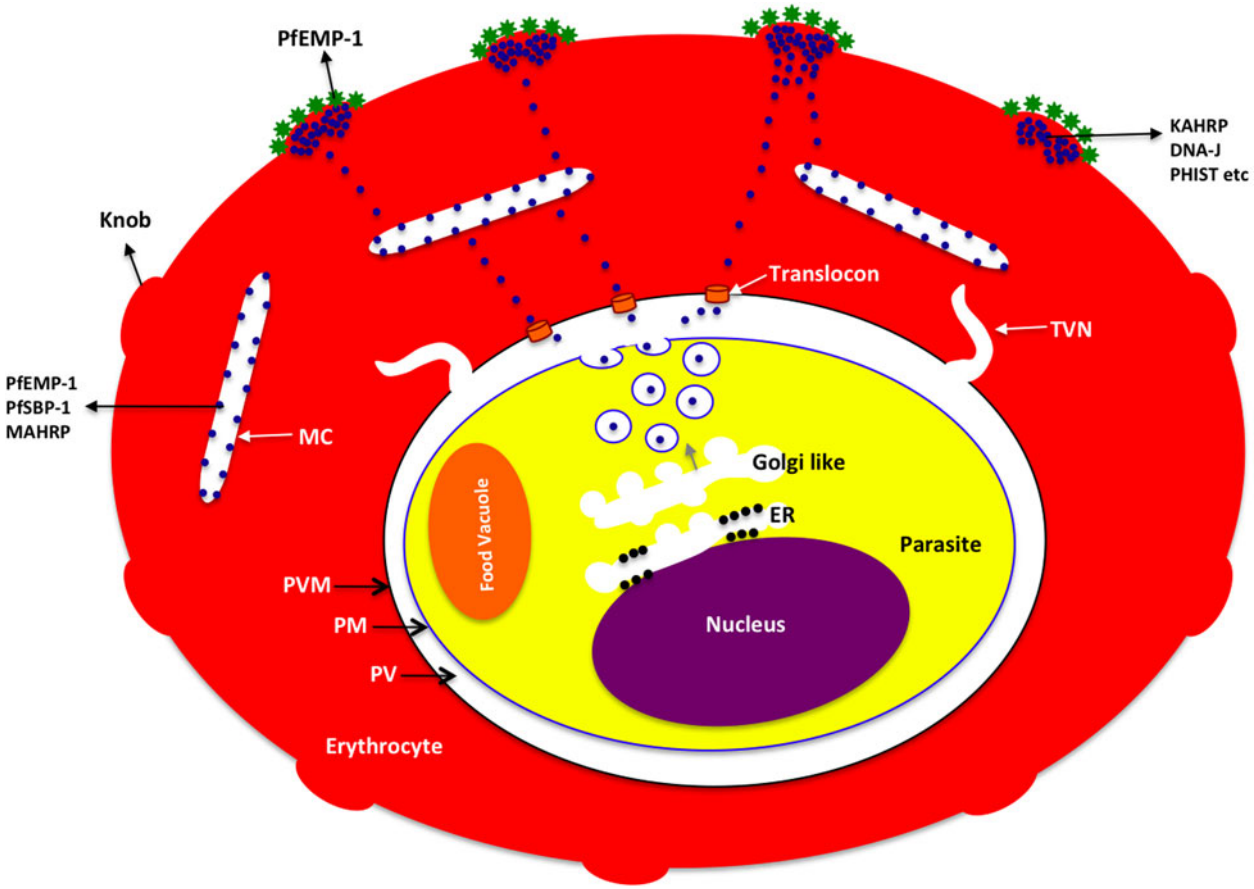

Fig. 1. Diagrammatic representation of channel-mediated soluble protein trafficking in the *P. falciparum*-infected erythrocyte. The figure shows exported proteins most likely exported via the secretory vesicle pathway. These proteins are recognized and processed (signal sequence and PEXEL) in Golgi-like bodies and then accumulate in small secretory vesicles that extend towards the parasite plasma membrane (PPM). After reaching PPM, a secretory vesicle becomes fused with PPM and discharges its soluble proteins in PV. The exported proteins then translocate the PVM with the help of a translocon present in PVM and enter its destinations in erythrocytes such as red cell cytoplasm, MCs, red cell membrane and the surface of red cells. TVN, tubulo-vesicular network; PV, parasitophorous vacuole; PVM, parasitophorous vacuole membrane; PM, parasite membrane; ER, endoplasmic reticulum; MCs, Maurer's clefts. The modelling channel-mediated trafficking of exported protein in infected erythrocyte is a modified version of Cooke *et al.* (2004).

erythrocyte is the interface of the parasite and the host-cell cytoplasm, i.e. the parasitophorous vacuole (PV) and its associated membrane (parasitophorous vacuole membrane (PVM)). In order to alter the host cell from a hostile environment to one that is conducive for parasite survival, parasite-encoded proteins have to traverse the PV and the PVM and enter the erythrocyte.

How, then, does the parasite achieve this? To understand protein trafficking beyond the PVM, two pathways of protein export have been proposed: one is vesicle mediated (Gormley *et al.* 1992; Foley and Tilley, 1998; Taraschi *et al.* 2001) and the other channel mediated (Gormley *et al.* 1992; Schatz and Dobberstein, 1996; Foley and Tilley, 1998; Schnell and Hebert, 2003). *Plasmodium falciparum* proteins thought to be exported via vesicles include PfEMP-1, PfSar1p, Pfsec31p, etc. (Gormley *et al.* 1992; Ansorge *et al.* 1996; Trelka *et al.* 2000; Taraschi *et al.* 2001, 2003), while soluble proteins such as KAHRP, PHIST, MESA, PfEMP-3, FIKK kinase, etc. are thought to be exported via the channel pathway (Hiller *et al.* 2004; Marti *et al.* 2004; Sargeant *et al.* 2006). The channel-mediated protein export pathway in *P. falciparum* is discussed here in detail. First, a signal sequence directs the protein for export to the lumen of the endoplasmic reticulum (ER) where the export signal is recognized and processed for entry into the secretory pathway. Through this secretory pathway, the protein crosses the PV and then the PVM through translocons present in the membrane (de Koning-Ward *et al.* 2009). The protein then enters the erythrocyte cytosol and is finally directed to its destination, which may be the host-cell cytosol, Maurer's clefts (MCs), the erythrocyte membrane or the surface of the erythrocyte (Fig. 1) (Cooke *et al.* 2004).

Recently, several key issues in protein export have been elucidated. These include the identification of an export signal (Hiller *et al.* 2004; Marti *et al.* 2004),

the mechanism by which the export signal is processed (Chang *et al*. 2008; Boddey *et al*. 2009, 2010; Russo *et al*. 2010; Bhattacharjee *et al*. 2012), the physical nature of exported proteins in the PV (Gehde *et al*. 2009), the translocon machinery (de Koning-Ward *et al*. 2009) and the role of MCs in directing proteins to the surface of the host cell (Bhattacharjee *et al*. 2008). Based on these breakthroughs, the channel-mediated protein export pathway has been modelled (Fig. 1) and advances in our current understanding of the various steps involved in protein trafficking are discussed in detail below.

Identification of the export signal and prediction of exported proteins

A major advance in our understanding of the protein trafficking mechanisms of malaria parasites was the identification of an export signal sequence, and the use of this to predict which proteins the parasite exports (Hiller *et al*. 2004; Marti *et al*. 2004). These studies revealed a consensus conserved amino acid motif downstream of the N-terminal sequences of many exported proteins, which was termed the *Plasmodium* export element (PEXEL) or the vacuole transport signal (VTS) (Hiller *et al*. 2004; Marti *et al*. 2004), which is conserved structurally and functionally across the *Plasmodium* species infecting humans and birds (Marti *et al*. 2004). The PEXEL/VTS motif generally lies within a 20–60 amino-acid stretch downstream of the N-terminal sequence of many exported proteins (Hiller *et al*. 2004; Marti *et al*. 2004; Sargeant *et al*. 2006) (Fig. 2). It consists of five amino acids RxLxE/Q/G, of which arginine (R) and lysine (L) are essential for recognition whereas the fifth amino acid glutamate (E) is not essential and can be replaced with glutamine (Q) (frequently) or glycine (G) (rarely) (Hiller *et al*. 2004; Marti *et al*. 2004) (x could be any amino acid). Further, the PEXEL processed proteins get acetylated at the new N-terminus, which appears to be crucial for recognition and transport of exported proteins to red cells (Chang *et al*. 2008; Boddey *et al*. 2009). A recent study has identified that many exported proteins can have a relaxed PEXEL motif (RxLxxE), which is functional and processed by the same mechanism used for canonical PEXEL (Boddey *et al*. 2013). Based on the updated information of the relaxed PEXEL motif, ExportPred version 1.0 (Sargeant *et al*. 2006) has been re-designed (version 2.0), which identified 73 additional exported proteins from the falciparum genome (Boddey *et al*. 2013).

The PEXEL/VTS motif does not appear to be essential for a protein to enter the export pathway, however, as several exported proteins do not possess such a motif. The exported proteins targeted to the host-cell cytoplasm that lack a well-defined

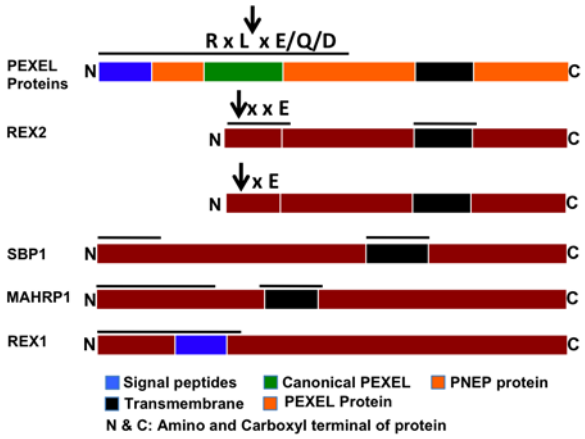

Fig. 2. Diagrammatic representation of *P. falciparum* PEXEL and PNEP protein structure. The figure represents a typical structure of an exported protein. The arrow indicates ER resident enzyme Plasmepsin-V that cleaves the PEXEL motif. Scenarios of export of PEXEL-negative exported proteins (PNEPs) are also presented. The bar above the colour indicates hydrophobic regions potentially involved in export and the figure is a modified version of Spielmann and Gilberger (2010).

PEXEL/VTS motif are denoted as PEXEL negative export proteins (PNEPs). These PNEPs include skeleton binding protein-1 (SBP-1), membrane-associated histidine-rich protein-1 (MAHRP-1), and ring export protein-1 and -2 (REX-1 and REX-2) (Blisnick *et al*. 2000; Spycher *et al*. 2003; Hawthorne *et al*. 2004; Spielmann *et al*. 2006; Haase *et al*. 2009; Saridaki *et al*. 2009), which are all resident proteins of MCs. The PNEPs (REX-2, SBP-1 and MAHRP-1) contain a conserved transmembrane domain but lack a signal sequence, whereas REX-1 has a recessed signal sequence (Dixon *et al*. 2008). These PNEPs have ER intermediates, suggesting export via a classical secretory pathway (Spycher *et al*. 2006; Dixon *et al*. 2008; Saridaki *et al*. 2008; Haase *et al*. 2009). It is very likely that processing of a signal peptide could also generate a similar N-terminus of PNEPs as generated by Plasmepsin-V; therefore, it has been proposed (Spielmann and Gilberger, 2010) that following processing by a signal peptidase, the trafficking of PNEPs may converge with PEXEL proteins at the translocon. The functional evaluation of *Plasmodium* export signals in *Plasmodium berghei* suggests that there may be multiple pathways of protein export for PEXEL and PNEP proteins in non-falciparum malaria parasites (Sijwali and Rosenthal, 2010). Based on the quantity of PEXEL/VTS positive proteins, it is apparent that the majority of proteins exported by *P. falciparum* are PEXEL/VTS positive, whereas in non-falciparum *Plasmodium* species, it is the PEXEL/VTS-independent proteins that make up the majority of the exportome (Table 1). The most well-characterized example of PEXEL/VTS-independent export in

Table 1. Canonical and non-canonical PEXEL motifs in exported proteins from *Plasmodium* species

Plasmodium species	Gene family	No. of genes	Canonical PEXEL[a]			Non-canonical PEXEL				
			RxLxE	RxLxD	RxLxQ	RxLxxE[b]	KxLxE	RxIxE	KxLxD	RxIxD
P. vivax	*Vir*	222	16 (7)	12 (4)	6 (1)	8 (2)	81 (21)	13 (3)	126 (25)	15 (1)
P. cynomolgi	*Cyir*	476	36 (17)	24 (7)	6 (0)	18 (7)	137 (56)	34 (20)	194 (68)	16 (7)
P. knowlesi	*SICAVAR*	242	25 (3)	20 (3)	12 (1)	19 (5)	141 (34)	103 (7)	107 (27)	8 (1)
P. knowlesi	*Kir*	60	11 (1)	6 (2)	5 (1)	4 (0)	25 (4)	6 (2)	42 (8)	7 (0)
P. berghei	*Bir*	199	7 (0)	6 (0)	1 (0)	0	96 (39	3 (2)	65 (5)	2 (1)
P. chabaudi	*Cir*	192	7 (1)	10 (4)	1 (0)	2 (2)	44 (6)	6 (1)	88 (11)	3 (0)
P. yoelii	*Yir*	785	24 (2)	14 (1)	3 (0)	9 (0)	237 (13)	11 (1)	380 (16)	16 (6)
P. falciparum	*Rifin*	185	146 (146)	1 (1)	2 (0)	3 (1)	143 (6)	3 (0)	85 (2)	2 (0)
P. falciparum	*Stevor*	41	13 (12)	1 (0)	29 (29)	0	31 (15)	0	8 (5)	0
P. falciparum	*RESA* like	71	38 (38)	12 (5)	11 (7)	12 (2)	29 (2)	4 (1)	19 (1	6 (0)
P. falciparum	*PHIST*	67	32 (31)	12 (6)	10 (7)	12 (3)	29 (2)	6 (1)	19 (1)	4 (0)
P. cynomolgi	*RESA/RAD*	57	28 (23)	8 (5)	6 (1)	5 (2)	15 (1)	4 (0)	10 (1)	3 (1)
P. falciparum	*Surfin*	11	1 (0)	7 (0)	1 (0)	2 (2)	10 (1)	8 (1)	11 (3)	4 (0)
P. falciparum	*Var*	105	64 (0)	16 (0)	15 (0)	12 (0)	83 (10)	31 (2)	64 (24)	15 (1)

Data obtained from the *Plasmodium* genome database (www.plasmodb.org). Canonical and non-canonical PEXEL motifs in the most likely exported proteins were estimated using the sequence pattern search tool (http://www-archbac.u-psud.fr/genomics/patternSearch.html). Number indicates number of paralogs in a gene family. Values in parentheses indicate the actual number of gene family members having a defined canonical or non-canonical PEXEL motif.

[a] Canonical PEXEL cleaved by Plasmepsin-V.

[b] Non-canonical PEXEL, which is also processed by Plasmepsin-V.

a non-*P. falciparum* malaria parasite is the export of the variant surface proteins of *P. vivax*, *Plasmodium cynomolgi*, *Plasmodium knowlesi* and *Plasmodium yoelii*/*P. berghei*/*Plasmodium chabaudi* (Table 1). Only a few members of the above variant gene families (Table 1) possess a canonical PEXEL/VTS motif near their signal sequences. *Plasmodium vivax* VIR proteins provide direct evidence for their export at the surface of infected red blood cells (iRBC; Bernabeu *et al*. 2012; Lopez *et al*. 2013). Indirect evidence for export of VIR proteins can be inferred from the structural similarity of VIR subfamilies A and D (Merino *et al*. 2006) with the exported proteins of *P. falciparum* SURFIN and two transmembrane (Pf2TM) proteins respectively that lack a canonical PEXEL (Sam-Yellowe *et al*. 2004; Winter *et al*. 2005; Alexandre *et al*. 2011). This suggests that the non-falciparum *Plasmodium* species may have different mechanisms for the export of proteins necessary for host-cell remodelling and virulence, and corroborates a hypothesis that canonical PEXEL/VTS exported proteins have undergone lineage-specific expansion in the falciparum parasite (Pick *et al*. 2011). Further, it is unclear whether the greater abundance of canonical PEXEL/VTS proteins in *P. falciparum* is due to the fact that *P. falciparum* requires a high degree of host-cell remodelling compared with non-falciparum malaria parasite species, and so exports more proteins, or to the fact that protein export in the non-falciparum species is mainly mediated through PEXEL/VTS-independent pathways (Pick *et al*. 2011).

Recognition and processing of the export signal

Canonical PEXEL/VTS-mediated protein export requires the recognition and processing of the export signal in the ER, the first step of entry of exported proteins into the trafficking/secretory pathway. The canonical PEXEL/VTS motif was initially thought to mediate the trafficking of exported protein across the PV; however, it was later identified as a cleavage site for a parasite protease within the ER (Chang *et al*. 2008; Boddey *et al*. 2009). The cleavage of the PEXEL/VTS motif in exported proteins directs access to the host-cell cytosol (Chang *et al*. 2008; Boddey *et al*. 2009). The PEXEL/VTS cleaving protease has been identified as Plasmepsin-V, an aspartic protease localized in the lumen of ER (Klemba and Goldberg, 2005), by two independent studies conducted with *P. falciparum* (Boddey *et al*. 2010; Russo *et al*. 2010). Plasmepsin-V recognizes the conserved PEXEL/VTS sequence (RxLxE/Q/D) and specifically cleaves between the lysine (L) and glutamate (E) residues (Fig. 2). The cleaved fragment carries an xE/Q/D at the N-terminal of the processed protein that is further N-acetylated (Chang *et al*. 2008; Boddey *et al*. 2009) and then recruited into the secretory pathway (Chang *et al*. 2008). The conservation of the xE/D/Q residue in a processed protein seems essential for export but not for processing (Boddey *et al*. 2009). Plasmepsin-V can process canonical PEXEL and relaxed PEXEL as well, such as RxLxxE (Boddey *et al*. 2013), indicating that this export signal cleaver is capable of processing a larger number of exported proteins (>400). However, genome analysis of *Plasmodium* species reveals several fixed patterns (relaxed PEXEL) in the N-terminus of various exported proteins (Table 1). Assuming that these non-canonical PEXEL could be processed by a parasite, as these are exported proteins, we can expect that the parasite might have additional ER resident proteases other than the well-known canonical PEXEL cleaver Plasmepsin-V.

Physical nature of exported proteins in the PV

Shared physical characteristics of exported proteins that traverse the parasite membrane (PM), PV and PVM may offer clues to aid the elucidation of the export/secretory pathway. It has been shown that exported proteins traverse the PV in an unfolded form (Gehde *et al*. 2009) (Fig. 3). The study was performed with a transgenic parasite expressing a chimeric protein in which a functional export signal is fused to green fluorescent protein (GFP) and to murine dihydrofolate reductase (mDHFR). In the presence of WR99210, all chimeric GFP–mDHFR fusion proteins were found to be accumulated in the PV; on the removal of WR99210, successful entry of the chimeric protein was observed in the host-cell cytoplasm. These unfolded proteins must then be refolded in the iRBC and then trafficked to their final destination (Gehde *et al*. 2009). This study confirms that exported proteins traverse the PV and PVM in an unfolded form and further underlines the putative role of heat shock proteins and molecular chaperones in unfolding exported proteins for successful translocation and refolding in the iRBC. Proteome analysis of *P. falciparum* revealed that the PV and iRBC are enriched with a number of heat shock proteins (Florens *et al*. 2002, 2004; Nyalwidhe and Lingelbach, 2006; Acharya *et al*. 2007). Genome sequence analysis has revealed a large repertoire of exported proteins in *P. falciparum* that are either heat shock proteins (Hiller *et al*. 2004; Marti *et al*. 2004; Nyalwidhe and Lingelbach, 2006; Shonhai *et al*. 2007) or contain the DNA-J domain (which encodes for a molecular chaperone), for example, the seven members of the PHIST-b protein and Hsp40 families (Sargeant *et al*. 2006; Acharya *et al*. 2007, 2012), and ring-infected erythrocyte surface antigen (RESA) protein (Favaloro *et al*. 1986). These proteins emphasize the importance of chaperone molecules in malaria parasite protein export pathways, and offer a relatively unexplored avenue for exportome research.

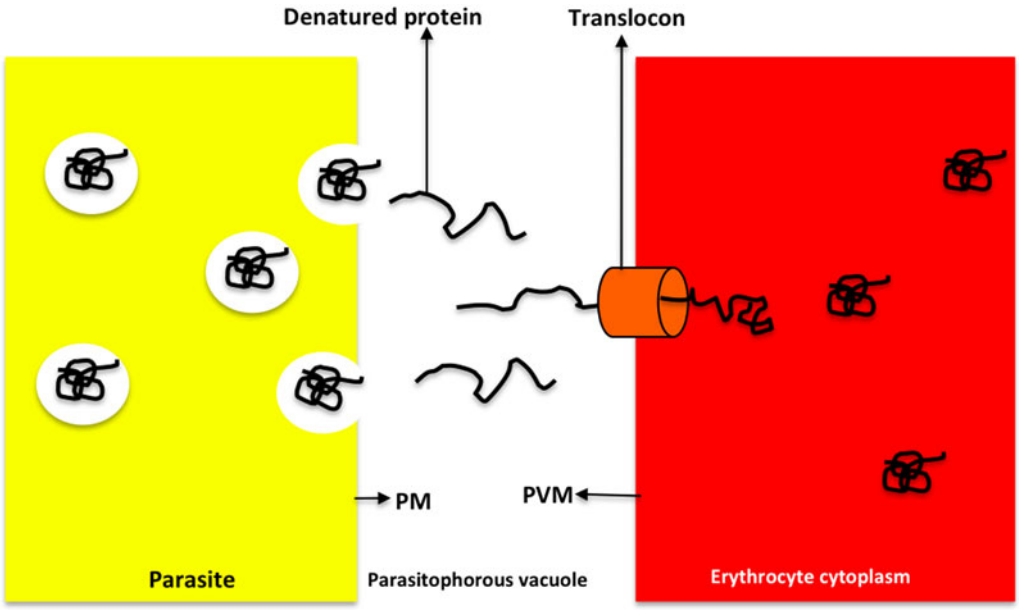

Fig. 3. Schematic representation of an exported protein in the PV in a *P. falciparum*-infected erythrocyte. Exported proteins enter PV, get unfolded and cross PVM and enter erythrocyte cytoplasm with the help of translocons. In erythrocyte cytoplasm, exported proteins first get refolded with the help of heat shock protein and then reach subcellular locations in erythrocytes. PVM, parasitophorous vacuole membrane; PM, parasite membrane. This image showing translocation of exported protein from PVM is drawn on the basis of work by Gehde *et al*. (2009).

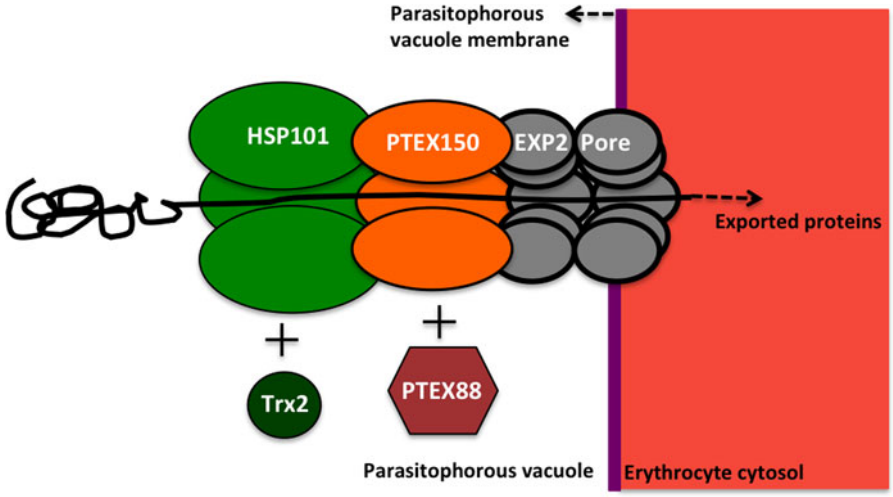

Fig. 4. Molecular composition and structure of the translocon in the PVM of the *P. falciparum*-infected erythrocyte. PTEX150, *Plasmodium* translocon of exported protein-150; PTEX88, *Plasmodium* translocon of exported protein-88; TRX-2, thioredoxin-2; HS101, heat shock protein-101; EXP2, exported protein-2. This figure is a modified version of work by de Koning-Ward *et al*. (2009) and Bullen *et al*. (2012).

Identification of translocon machinery in the PVM

Channel-mediated delivery is a translocon. As channel-mediated protein trafficking necessitates machinery within the PVM to facilitate protein movement through the membrane, so there must exist translocon(s) embedded within the PVM (Schatz and Dobberstein, 1996; Schnell and Hebert, 2003). Such a translocon has recently been described in *P. falciparum* (de Koning-Ward *et al*. 2009). It appears that the *P. falciparum* translocon machinery consists of a complex of parasite-encoded proteins embedded within the PVM, termed the *Plasmodium* translocon of export protein (PTEX). The PTEX complex is an ATP-powered machine and consists of two PTEX proteins (PTEX150 and PTEX88), a heat shock protein (Hsp101), thioredoxin protein-2 (TRX2) (Matthews *et al*. 2013) and parasite export protein-2 (EXP2) (de Koning-Ward *et al*. 2009; Bullen *et al*. 2012; Riglar *et al*. 2013). A recent study has shown that a homodimer of EXP2 oligomerizes, forms a pore-like shape and attaches to the remainder of the PTEX complex in PVM (Bullen *et al*. 2012) (Fig. 4). Since *P. falciparum* exports

hundreds of highly diverse remodelling and virulence-associated proteins, it is proposed that the parasite may utilize structurally related PVM translocons that may have different accessory proteins or exported proteins may use different cargo. Thus, functional dissection of translocon complexes and modelling of translocon machineries would provide further insights into protein trafficking in *P. falciparum*.

Trafficking of exported proteins within the erythrocyte cytoplasm

The fate of exported proteins and the mechanisms behind their journey following their exit from the PVM remains relatively poorly understood. A number of studies have suggested an important role of MCs in the sorting and further trafficking of exported proteins to the surface of infected erythrocytes (Lanzer *et al*. 2006; Wickert and Krohne, 2007; Bhattacharjee *et al*. 2008; Tilley *et al*. 2008). MCs are parasite-induced, flattened membranous structures scattered throughout the cytoplasm of infected erythrocytes (Spycher *et al*. 2006). However, it remains to be elucidated how the sorting of proteins to the RBC membrane and the surface of the iRBC occurs following protein traversal of the PVM. The resident proteins of MCs have been shown to play an important role in the sorting and trafficking of PfEMP-1 from MCs to the erythrocyte surface. These resident proteins include Pf332, PfEMP-3, PfSBP-1 and MAHRP-I (Waterkeyn *et al*. 2000; Spycher *et al*. 2003; Maier *et al*. 2007; Hodder *et al*. 2009). Previously, it was believed that MCs are responsible for the display of exported proteins on the surface of infected erythrocytes by a mechanism in which MCs ultimately merge with the host-cell membrane (Trelka *et al*. 2000). Later studies described the role of MCs as a secretory organelle that accumulates parasite proteins and delivers them to the surface of the erythrocyte (Bhattacharjee *et al*. 2008). Further, a large-scale gene knockout study confirmed their role in the trafficking of PfEMP-1 to the surface of the infected erythrocyte (Maier *et al*. 2008). The export of PfEMP-1, one of the most well-characterized virulence-associated proteins, to the surface of the infected erythrocyte has been proposed to be completed in three phases, early, middle and late, wherein each phase involves a number of other parasite-encoded proteins assisting the trafficking through protein–protein interactions (Maier *et al*. 2008). Other parasite-encoded proteins involved in PfEMP-1 trafficking to the surface of infected erythrocytes have been reviewed by Sam-Yellowe (2009). Further investigations are required in order to fully understand the mechanisms behind the transport of proteins beyond the PVM to their final destination in the host-cell cytoplasm and on to the RBC surface.

EXPANSION OF GENE FAMILIES INVOLVED IN PROTEIN TRAFFICKING

The number of predicted exported proteins is far fewer in non-falciparum malaria species than in *P. falciparum* when algorithm-based predictions are used (Pick *et al*. 2011). The majority of predicted exported proteins belong to 27 well-characterized gene families, including *stevor, pfemp-1, rifin, fikk kinases, surfin* and *pf2tm* and 21 other novel gene families (Sargeant *et al*. 2006; Boddey *et al*. 2013). Some of the major novel exported protein-encoding gene families are PHIST, DNA-J and hydrolase proteins (Sargeant *et al*. 2006). The majority of predicted exported proteins are hypothetical and require further functional characterization and annotation. Furthermore, phylogenetic studies suggest the occurrence of lineage-specific expansion of the *phist, DNA-J* and *FIKK kinases* gene families in the *P. falciparum* genome (Ward *et al*. 2004; Sargeant *et al*. 2006). One member of the *phist-b* gene family has been shown to be involved in knob formation (Maier *et al*. 2008; Acharya *et al*. 2012).

The expansion of various gene families in *P. falciparum* but not in non-falciparum malaria species suggests that radiation of these gene families may have shaped the specific pathogenesis of this parasite (Sargeant *et al*. 2006; Pick *et al*. 2011). The high virulence of *P. falciparum* compared with non-falciparum malaria species is mainly mediated by the localization of PfEMP-1 on the surface of infected erythrocytes. Studies of the export of PfEMP-1 beyond the PVM have been carried out using the PfEMP-1 protein (Var2CSA) expressed by the CS-2 cloned line of *P. falciparum* that causes placental malaria (Salanti *et al*. 2004). The genome of this parasite has 59 different PfEMP-1 proteins expressed in a mutually exclusive way so that a single PfEMP-1 protein is expressed during each schizogonic cycle in the blood (Dzikowski *et al*. 2006*a*, *b*). This suggests that the actual number of exported proteins required for the successful expression of PfEMP-1 at the surface of the erythrocyte may be larger than currently thought. A large-scale gene knockout study has described the involvement of various PHIST proteins in PfEMP-1 trafficking as well as in the modification of erythrocyte membrane rigidity (Maier *et al*. 2008). Recently, a single PHIST protein has been shown to have vital interaction with the ATS domain of PfEMP-1 (Mayer *et al*. 2012), suggesting a putative role for PHIST proteins in trafficking parasite-encoded proteins in the infected erythrocyte. Therefore, the lineage-specific expansion of PHIST and other export families (Sargeant *et al*. 2006) may be a specific requirement of

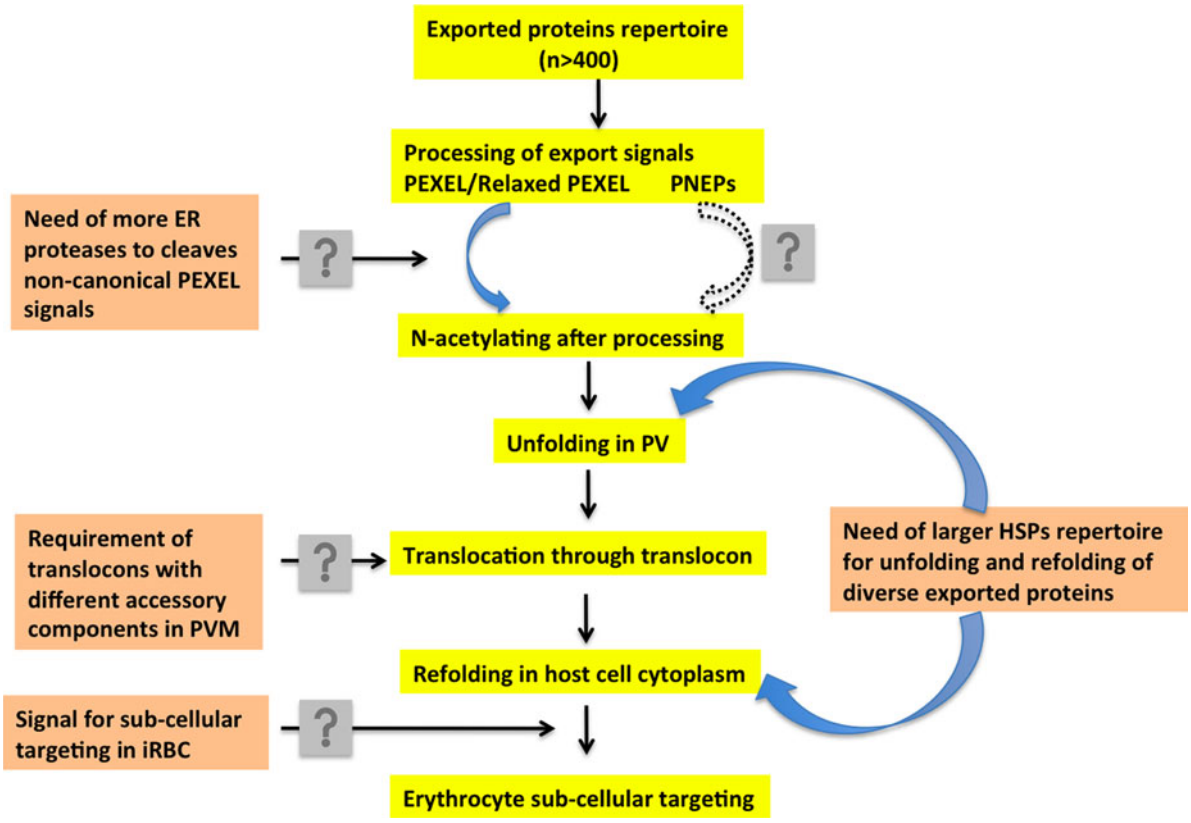

Fig. 5. Diagrammatic representation of the protein export pathway in *P. falciparum*-infected red cells. The figure shows knowledge gaps in the protein trafficking pathway, which are (1) requirement of additional ER peptidases to process a non-canonical PEXEL signal, (2) requirement of additional translocons that may have structurally related composition as of a well-known translocon, (3) characterization of heat shock proteins that helps unfolding of diverse exported proteins in PV and refolding in infected erythrocyte cytoplasm and (4) characterization of signal in exported proteins for subcellular targeting in infected erythrocytes.

P. falciparum relating to the trafficking of host-cell remodelling and virulence-associated proteins.

PERSPECTIVE

Key advances in analysing protein export beyond the PVM using parasite genetic modification and *in vivo* imaging technologies have resulted in a clearer understanding of protein trafficking pathways in *P. falciparum*. Despite these advances, critical parts of the parasite's protein transport mechanisms remain incompletely understood. Based on the current knowledge on protein trafficking in malaria-infected erythrocytes and exportome size, a schematic diagram of the protein trafficking pathway (Fig. 5) has been prepared that shows possible knowledge gaps. Many intriguing questions still remain to be answered. For example, does a parasite have multiple ER-resident proteases to process non-canonical PEXEL? Can translocons have different accessory proteins? How do unfolded exported proteins become refolded in the host-cell cytoplasm? How are PEXEL/VT-negative proteins exported beyond the PVM? Is expansion of gene families involved in protein trafficking related to exportome size of a malaria parasite?

The trafficking of parasite-encoded proteins beyond the parasite and into the RBC cytosol and onwards onto the RBC surface is, presumably, unique to malaria parasites. Further characterization of these processes could provide useful knowledge for the design of therapeutics as well as for the elucidation of a fascinating aspect of the biology of the malaria parasite.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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